

# Methods and means for delivering inhibitory RNA to plants and applications thereof

## Cross-Reference to Related Applications

SUB 5 17 This application is a continuation-in-part of U.S. Serial No. 09/294,022, filed April 20, 1999, the contents of which are incorporated herein by reference.

## Field of the invention.

10 This invention relates to the field of functional genomics in plants, more particularly it relates to methods for the further identification and isolation of a nucleic acid with a nucleotide sequence of interest in a collection of preselected nucleic acid sequences correlated with a particular trait, preferably an agronomical important trait, using a kit of viral RNA vectors which allow systemic

15 spread of all components of the kit in a plant, wherein one of the viral RNA vectors comprises a library of gene-silencing constructs for the preselected nucleic acid sequences. The invention also relates to a method for modulating, preferably reducing, particularly eliminating the expression of a selected nucleic acid sequence, using the viral vector kit, whereby one of the vectors comprises

20 a gene-silencing construct for the selected nucleic acid sequence. The latter method may be used for validating the function of a nucleic acid sequence whose expression is correlated with the presence or absence of a specific trait in plants, but with otherwise unknown function. Preferably, one of the viral RNA vector components of the kit is a vector derived from a satellite virus.

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## Background art

The recent, rapid expansion of available nucleic acid sequence information has necessitated the development of methods for identifying the function of nucleic

30 acid sequences, particularly transcribed nucleic acid sequences such as

expressed sequence tags, with unknown function, in an efficient and labor-cost effective way.

To identify the role of sequenced nucleic acids from plants of unknown function it is necessary to produce or identify plants in which those nucleic acids are either structurally or functionally inactivated. Plants wherein predetermined nucleic acid sequences are structurally inactivated can be generated using recombination technologies such as homologous recombination as described by Kempin et al. (1997) or using specific technologies such as the use of mixed duplex oligonucleotides (chimeraplasts) to generate specific mutations (as described in WO 96/22364 and WO 99/07865) . Alternatively, plants with a mutation in a predetermined nucleotide sequence can be identified by screening a saturated mutant library, such as but not limited to a T-DNA insertion library or a transposon insertion library (see e.g. Pereira and Aerts, 1998). These methodologies all require the generation of a large number of permanently altered plants, and thus are less amenable for application in high throughput methods. Moreover, the recovery of plants with recessive mutations in essential genes requires time-consuming breeding to maintain the plants in heterozygous state. Maintenance of dominant lethal mutations in essential genes is virtually impossible.

Plants with functionally inactivated predetermined nucleotide sequences can be generated in a straightforward way using methodologies wherein inhibitory RNA is generated, such as antisense or sense RNA.

The use of inhibitory RNA to reduce or abolish gene expression, also known as gene silencing, is well established in the art and is the subject of several reviews (e.g Baulcombe 1996, Stam et al. 1997, Depicker and Van Montagu, 1997). Several patent applications relate to the practical exploitation of gene silencing.

US 5,190,131 and EP 0 467 349 A1 describe methods and means to regulate or inhibit gene expression in a cell by incorporating into or associating with the

genetic material of the cell a non-native nucleic acid sequence which is transcribed to produce an mRNA which is complementary to and capable of binding to the mRNA produced by the genetic material of that cell.

5 EP 0 240 208 describes a method to regulate expression of genes encoded for in plant cell genomes, achieved by integration of a gene under the transcriptional control of a promoter which is functional in the host and in which the transcribed strand of DNA is complementary to the strand of DNA that is transcribed from the endogenous gene(s) one wishes to regulate.

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EP 0 223 399 A1 describes methods to effect useful somatic changes in plants by causing the transcription in the plant cells of negative RNA strands which are substantially complementary to a target RNA strand. The target RNA strand can be a mRNA transcript created in gene expression, a viral RNA, or other RNA present in the plant cells. The negative RNA strand is complementary to at least a portion of the target RNA strand to inhibit its activity *in vivo*.

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EP 0 647 715 A1 and US patents 5, 034,323, 5,231,020 and 5,283,184 describe methods and means for producing plants exhibiting desired phenotypic traits, by selecting transgenotes that comprise a DNA segment operably linked to a promoter, wherein transcription products of the segment are substantially homologous to corresponding transcripts of endogenous genes, particularly endogenous flavonoid biosynthetic pathway genes.

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25 W0 93/23551 describes methods and means for the inhibition of two or more target genes, which comprise introducing into the plant a single control gene which has distinct DNA regions homologous to each of the target genes and a promoter operative in plants adapted to transcribe form such distinct regions RNA that inhibits expression of each of the target genes.

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A major disadvantage of these technologies, which hampers the exploitation thereof in high throughput gene function discovery methods, is the intrinsic unpredictability and low occurrence of the gene silencing phenomenon.

- 5 Recently, Waterhouse et al. (1998) have described methods and means to make gene silencing in plants more efficient and predictable, by simultaneous expression of both sense and antisense constructs in cells of one plant. The sense and antisense nucleic acids may be in the same transcriptional unit, so that a single RNA transcript that has self-complementarity is generated upon  
10 transcription.

Hamilton et al. (1998) describe improved silencing e.g. of tomato ACC-oxidase gene expression using a sense RNA containing two additional upstream inverted copies of its 5' untranslated region.

- 15 WO 98/53083 describes constructs and methods for enhancing the inhibition of a target gene within an organism, involving the insertion into the gene silencing vector of an inverted repeat of all or part of a polynucleotide region within the vector.

- 20 It should be clear however, that the use of inhibitory RNA as a tool in reversed genetics analysis of gene function via high throughput methods, whereby the inhibitory RNA is generated from gene-silencing constructs which are stably integrated in the genome of transgenic plants, suffers from the same drawbacks  
25 as the methods wherein the nucleotide sequences are structurally inactivated.

EP 0 194 809 and US 5,500,360 suggest the use of viral RNA vectors to produce regulatory RNA such as anti-sense RNA.

- 30 Initial exploration of the use of viral vectors to deliver inhibitory RNA into cells of plants has been described by Chapman (1991). In this publication, gene silencing constructs comprising nucleotide sequences complementary to the

translated region of the GUS gene on a PVX derived viral vector were described. The experiments however, remained inconclusive as to whether gene silencing could be provoked using viral vectors for the production of inhibitory RNA.

5 WO 93/03161 is directed toward recombinant plant viral nucleic acids and to hosts infected thereby. The non-native nucleic acid sequence which is transcribed may be transcribed as an RNA which is capable of regulating the expression of a phenotypic trait by an anti-sense mechanism.

10 English et al., 1996 describe the suppression of the accumulation of a viral vector comprising a foreign nucleotide sequence in transgenic plants exhibiting silencing of nuclear genes comprising the same foreign nucleotide sequences, thus linking gene silencing and viral vectors, albeit in a reverse way as envisioned here.

15 Kumagai et al. 1995 (PNAS 92, 1679-1683) described the inhibition of phytoene desaturase gene by viral delivery of antisense RNA.

20 WO 95/34668 suggests the use of genetic constructs based on RNA viruses which replicate in the cytoplasm of cells to provide inhibitory RNA, either antisense or co-suppressor (sense) RNA.

25 Baulcombe et al. (1998) and Ruiz et al. (1998) describe virus-induced gene silencing of the endogenous phytoene desaturase gene (PDS) or of a green fluorescent protein transgene (GFP) in plants, using potato virus X derived vectors carrying inserts homologous to PDS and GFP, respectively. The authors further suggested that virus-induced gene silencing may develop into a novel assay of gene function, by introducing a fragment of the genome of a viral vector and inferring the function of the gene from the symptoms of the infected plants

30 exhibiting gene silencing.

The described methods for identification of the function of a gene with known nucleotide sequence however, have drawbacks and limitations. In the first place, the applicability of the mentioned viral RNA vector based gene silencing methods on larger scale is in practice limited to the identification of genes with essential functions or genes with macroscopically visible phenotypes. Secondly, all methods employ viral vectors which are capable of autonomous replication in plant cells and cell-to-cell movement, whereby care has to be taken not to inactivate the essential functions required for these functions. This may particularly be a disadvantage when tailoring these methods to the needs of particular plants, such as crop plants, by developing new viral vectors more apt for replication and systemic spread in the plants.

The prior art is thus deficient in the lack of efficient methods for large scale identification of the function of nucleic acids with known nucleotide sequence, or for the isolation of the genes of interest from a pool of genes with known nucleotide sequence, but unknown function.

### **Brief Description of the figures**

Figure 1 is a schematic representation of the viral RNA vectors used in the Examples. MP: movement protein; CP: coat protein; OAS: origin of assembly. The open reading frames are indicated by boxes. Each original viral genome is characterized by a specific pattern.

▧: tobacco mosaic virus; ▨ tobacco necrosis virus; ▩ satellite tobacco mosaic virus; ■ satellite tobacco necrosis virus.

### **Summary of the invention**

The invention provides a method for isolating genes involved in the determination of a trait or a phenotype of a plant species, comprising identifying a set of nucleic acids sequences of genes, whose expression is correlated with a trait of interest; creating a library of gene silencing constructs targeted or adapted to the nucleotide sequence of the identified nucleic acids in a viral RNA vector which is capable of replication inside plant cells and optionally, movement

between plant cells of a plant; infecting a collection of individual plants of the same plant species with the library of gene silencing constructs whereby each plant is infected with at least one member of the library; identifying a plant wherein the trait or phenotype is altered using an assay adapted to that trait or phenotype; and isolating the gene involved in the determination of the trait or phenotype in the plant species, from the library, based on the nucleotide sequence to which the gene silencing construct in the identified plant was targeted. Preferably the viral RNA vector is capable of autonomous replication inside plant cells and optionally autonomous movement between plant cells and particularly the viral RNA vector is derived from cowpea mosaic virus.

Alternatively, the viral RNA vector, is derived from a satellite RNA virus, preferably satellite tobacco mosaic virus or satellite tobacco necrosis virus, particularly it further comprises an origin of assembly of tobacco mosaic virus, and is capable of replication inside plant cells and optionally movement between plant cells when the required factors are supplemented *in trans*, preferably by infection with a helper virus, preferably tobacco mosaic virus or a helper virus which is derived from tobacco necrosis virus and comprises a gene encoding a coat protein gene of tobacco mosaic virus, and optionally, a movement protein of tobacco mosaic virus.

Gene-silencing constructs comprised within the viral RNA vector may comprise antisense RNA or sense RNA, preferably they may comprise an inverted repeat. Particularly the gene-silencing constructs comprise complementary stretch of at least 50, preferably at least 100 nucleotides of sense and antisense RNA. Especially preferred are gene-silencing constructs comprising at least two copies of part of the nucleotide sequences of the collection of nucleic acids, the copies being in inverted repeat.

It is another object of the invention to provide a method for the isolation or selection of a nucleic acid with a specific function from a collection of nucleic acids, wherein the collection of nucleic acids is characterized by the fact that

variation in the expression pattern of the nucleic acids is correlated with variation in a trait or phenotype of a plant harboring the nucleic acids comprising the steps of creating a library of gene silencing constructs targeted or adapted to the nucleotide sequence of the identified nucleic acids in a viral RNA vector which is capable of replication inside and optionally movement between plant cells; infecting a collection of plants with the library of gene silencing constructs, whereby each plant is infected with one member of the library; identifying plants with altered trait or phenotype using an assay adapted to the trait or phenotype under investigation; and optionally isolating the nucleic acid with the specific function from the identified plant with altered trait or phenotype.

It is yet another object of the invention to provide a method for determining the function encoded by a nucleic acid comprising a known nucleotide sequence in a plant, comprising the steps of providing a viral RNA vector derived from a satellite RNA virus, comprising a gene-silencing construct targeted to a gene comprising the known nucleotide sequence; infecting or inoculating the plant with the chimeric viral RNA vector and a corresponding helper virus or helper virus RNA; and identifying an altered trait or phenotype of the co-infected plant.

The invention further provides a method for isolating essential genes from a plant, comprising creating a library of random gene-silencing constructs, preferably by cloning random DNA fragments of the plant or by cloning random cDNA fragments, particularly duplicated cDNA fragments in inverted repeat in a cDNA copy of the viral RNA vector derived from a satellite RNA virus, preferably STMV or STNV, particularly a viral RNA vector comprising an origin of assembly of TMV; infecting a plant with individual members of the library and with a corresponding helper virus, preferably tobacco mosaic virus or a helper virus derived from tobacco necrosis virus and comprising the coat protein of tobacco mosaic virus and optionally the movement protein; identifying plants developing gene-silencing-construct-associated necrosis and optionally isolating the viral RNA vector from the necrotized tissue.



It is yet another object of the invention to provide a method for introduction of inhibitory RNA, preferably sense or antisense RNA, particularly inhibitory RNA comprising an inverted repeat, especially inhibitory RNA comprising a complementary stretch of at least 50, preferably at least 100 nucleotides of sense and antisense RNA, into plant cells, preferably into the cytoplasm of plant cells, comprising introducing into a plant cell, a viral RNA vector comprising the inhibitory RNA or comprising a chimeric nucleic acid which when transcribed yields the inhibitory RNA, wherein the viral RNA vector is derived from a satellite RNA virus, preferably STMV or STNV, particularly a STMV - derived or STNV derived RNA vector comprising an origin of assembly from tobacco mosaic virus; and introducing into the same plant cell, a corresponding helper virus, preferably tobacco mosaic virus or a chimeric helper virus derived from tobacco necrosis virus and comprising a coat protein gene of tobacco mosaic virus and optionally, a movement protein encoding gene of tobacco mosaic virus.

The invention further provides a kit for introduction of inhibitory RNA, preferably sense or antisense RNA, particularly inhibitory RNA comprising an inverted repeat, especially inhibitory RNA comprising a complementary stretch of at least 50, preferably at least 100 nucleotides of sense and antisense RNA in the cytoplasm of a plant cell comprising 1) a viral RNA vector derived from a satellite RNA virus, comprising the inhibitory RNA or which comprise a chimeric nucleic acid which when transcribed yields the inhibitory RNA; and 2) a corresponding helper virus.

A particularly preferred kit comprises 1) a viral RNA vector derived from a satellite tobacco mosaic virus, comprising an origin of assembly of tobacco mosaic virus, further comprising or encoding the inhibitory RNA; and 2) a corresponding helper virus derived from a tobacco mosaic virus.

Another particularly preferred kit comprises 1) a viral RNA vector derived from satellite tobacco necrosis virus, especially STNV-2 or STNV-C and comprising an origin of assembly of tobacco mosaic virus further comprising or encoding the

inhibitory RNA; and 2) a corresponding helper virus derived from tobacco necrosis virus, particularly TNV-A or TNV-D, which comprises a coat protein gene of tobacco mosaic virus and optionally a movement protein gene of tobacco mosaic virus.

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### **Detailed description of preferred embodiments.**

The following definitions apply throughout this application, unless otherwise specified.

10 As used herein "comprising" is to be interpreted as specifying the presence of the stated features, integers, steps or components as referred to, but does not preclude the presence or addition of one or more features, integers, steps or components, or groups thereof. Thus, e.g., a nucleic acid or protein comprising a sequence of nucleotides or amino acids, may comprise more nucleotides or  
15 amino acids than the actually cited ones, i.e., be embedded in a larger nucleic acid or protein. A chimeric gene comprising a DNA region which is functionally or structurally defined, may comprise additional DNA regions etc.

As used herein, "a trait of a plant" indicates a phenotype which is the combined  
20 result of the coordinated expression of a number of genes. Typical traits include yield, heterosis, drought-resistance, stress-resistance, high or low temperature-resistance, vigor, seed yield, plant habitat, architecture etc. Typically, a trait of a plant is named after its intended appearance.

25 A used herein a "phenotype" of a plant refers to any quantitative or qualitative characteristic of that plant, be it morphological (including macroscopic and microscopic characteristics), biochemical (including the presence, absence or concentration of particular metabolites or molecules) functional or other.

30 The term "gene" means any DNA or RNA fragment comprising a region (the "transcribed region") which is transcribed into a RNA molecule (e.g., a mRNA) in a cell, operably linked to suitable regulatory regions, e.g., a plant-expressible

promoter. A gene may thus comprise several operably linked fragments such as a promoter, a 5' leader sequence, a coding region, and a 3' region comprising a polyadenylation site. A plant gene endogenous to a particular plant species or virus (endogenous plant or virus gene) is a gene which is naturally found in that plant species or virus, or which can be introduced in that plant species by breeding techniques such as conventional breeding techniques. A chimeric gene is any gene which is not normally found in a plant species or, alternatively, any gene in which the promoter is not associated in nature with part or all of the transcribed DNA region or with at least one other regulatory region of the gene.

The term "expression of a gene" refers to the process wherein a DNA or RNA region which is operably linked to appropriate regulatory regions, particularly to a promoter, is transcribed into an RNA which is biologically active i.e., which is either capable of interaction with another nucleic acid or which is capable of being translated into a biologically active polypeptide or protein. A gene is said to encode an RNA when the end product of the expression of the gene is biologically active RNA, such as e.g. an antisense RNA, a ribozyme or a replicative intermediate. A gene is said to encode a protein when the end product of the expression of the gene is a biologically active protein or polypeptide. In addition to the above defined elements, a gene may further comprise elements for cap-independent translation such as an internal ribosome entry sequence or the first and second translation enhancing elements as defined in WO 97/49814.

As used herein the terms "gene-silencing" or "inhibitory" are not to be interpreted as meaning a complete abolishing of the expression of the target gene(s) but also includes any reduction in expression, measured either as a reduction in transcription and/or translation, as a reduction in the accumulation of transcripts or translation products such as proteins, or as a reduction in the phenotypic expression of the target gene.

The term "reduction of phenotypic expression" refers to the comparison of the phenotypic expression of the nucleic acid of interest in the eukaryotic cell in the presence of the inhibitory RNA or gene-silencing constructs of the invention, to the phenotypic expression of the nucleic acid of interest in a similar eukaryotic cell in the absence of the inhibitory RNA or gene-silencing constructs of the invention. The phenotypic expression in the presence of the inhibitory RNA of the invention should thus be lower than the phenotypic expression in absence thereof, preferably be only about 25%, particularly only about 10%, more particularly only about 5% of the phenotypic expression in absence of the inhibitory RNA, especially the phenotypic expression should be completely inhibited for all practical purposes by the presence of the inhibitory RNA or the gene-silencing construct encoding such an RNA.

A reduction of phenotypic expression of a nucleic acid where the phenotype is a qualitative trait means that in the presence of the inhibitory RNA, the phenotypic trait switches to a different discrete state when compared to a situation in which such inhibitory RNA is absent. A reduction of phenotypic expression of a nucleic acid may thus also be measured as a reduction in transcription of (part of) that nucleic acid or reduction in the level of transcript, a reduction in translation of (part of ) that nucleic acid or reduction in the level of translation products, or a reduction of the effect the presence of the transcribed RNA(s) or translated polypeptide(s) have on the eucaryotic cell or the organism, and will ultimately lead to altered phenotypes. It is clear that the reduction in phenotypic expression of a nucleic acid of interest, may be accompanied by or correlated to an increase in a phenotype or trait.

In one embodiment of the invention, a method is provided to identify and isolate genes involved in the determination of a trait or a phenotype of a plant. To this end, nucleic acid sequences are identified whose expression is correlated with the trait and/or phenotype of interest. Methods and means are available in the art for the almost simultaneous identification and/or isolation of a large number, if not the predominant part, of nucleotide sequences whose expression,

particularly whose transcription, is influenced subsequent to a stimulus corresponding to the trait to be investigated, in comparison with expression of these nucleotide sequences in a control plant. Such methods include but are not limited to differential display methods, such as the gel -based RNA differential display methods described by (Prahasar et al. 1996)

As a result of these methods, a collection of at least partially characterized nucleotide sequences with altered expression in response to a particular stimulus is identified. Typically, however, the application of such methods does not allow to discriminate between genes whose altered expression is directly caused by the stimulus, and those who are further downstream in the chain of events and are only indirectly influenced by the stimulus and a further selection amongst the obtained collection of nucleotide sequences will be required. Even less do these methods allow to predict whether the inverse relationship also holds, i.e. whether influencing the expression of genes with particular nucleotide sequences, identified in the above mentioned way, also influences the trait of interest. A further validation of the obtained sequences is thus required, and preferably one which immediately verifies the above mentioned inverse relationship. To achieve this goal in a efficient and cost-effective way, a library of gene-silencing constructs may be created in a viral RNA vector which is capable of replication inside plant cells and movement between cells of a plant, so as to assure an efficient systemic spread within a plant which has been infected with a clone of such a library. The created library of gene-silencing constructs comprised within a viral RNA vector is then used to infect a representative number of plants in such a way that at each plant is infected by at least one member (one clone) of the library. The infected plants can than be analyzed to identify those plants which exhibit alterations in the trait under investigation, using an assay which is adapted to the trait under investigation. It is clear that this fine-tuning of assay and trait under investigation may be an important advantage over the existing methods for high throughput analysis, particularly when analyzing traits and/or phenotypes which do not result in macroscopically visible alterations, such as but not limited to modifications in specific metabolic pathways or alterations which are only detectable under

specific conditions (e.g. heat, stress, drought-tolerance, pathogen-infection, application of specific herbicides or insecticides etc.).

5 The subset of nucleic acid sequences may also be identified on the basis of the presence of a particular signature characteristic of a class of proteins, such as but not limited to a kinase-specific domain, a binding motif etc.

10 The gene-silencing construct may then be isolated from the library or from the plant exhibiting the altered trait or phenotype, and be used to isolate the corresponding gene based on the nucleotide sequence towards which the gene silencing construct was targeted.

15 In a preferred embodiment, the viral RNA vector is capable of autonomous replication inside plant cells and autonomous movement between plant cells. Such viral RNA vectors are known in the art and may be based on Potato Virus X as described e.g. in WO 93/03161, WO95/34668, Ruiz et al. (1998)

20 In a particularly preferred embodiment, the used viral RNA vector is derived from cowpea mosaic virus. Wellink et al. (1998) have described the use of a viral vector derived from this RNA virus for the expression of GFP in plants and demonstrated that the virus and RNA vectors derived thereof have an excellent capacity for spreading throughout an infected plant, particularly *Nicotiana benthamiana*. CPMV is an icosahedral virus with a bipartite RNA genome, consisting of a longer and a shorter RNA. Wellinck et al. have demonstrated that  
25 it is possible to incorporate extra genetic information in the shorter RNA, by inserting the GFP coding region in frame into the viral encoded polyprotein. For the purpose of the herein described methods, it is preferred that the inhibitory RNA encoding nucleic acid be inserted downstream of the polyprotein encoding open reading frame.

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The inventors have obtained for the first time indications that gene-silencing may be obtained in plant cells such as protoplasts, using a viral vector derived from

a satellite virus comprising a  $\beta$ -1,3-glucanase coding region, in a co-infection experiment with a helper virus of the satellite virus

In another preferred embodiment, the viral RNA vector is capable of replication and cell-to-cell movement, only when the required functions are provided *in trans*. Particularly preferred, is the use of a satellite virus derived RNA vector, which can replicate in plant cells and spread throughout the plant, when a corresponding helper virus is present.

As used herein, "a satellite virus" indicates an RNA virus, preferably a single stranded RNA virus, the RNA genome of which is capable of replicating in a plant cell and being encapsidated by coat protein molecules to form a virus particle or virion, only when provided externally with any number of required essential functions therefor. By "externally provided" is meant that such functions are not encoded by the satellite viral genome. Satellite viruses thus depend upon external provision of essential functions, and may lack the capacity to encode functional replicase, movement protein, or other essential functions required to complete their life cycle inside a plant cell. In a natural situation, such essential functions are usually provided by an autonomously replicating virus or so-called helper virus.

Satellite viruses useful for the present invention may include wild type isolates, but also encompassed by this definition are variants which result in reduced or minimal symptoms when infected on a host plant, particularly when co-inoculated with a corresponding helper virus. The definition also includes synthetic satellite viruses such as defective viruses and chimeric satellite viruses.

A "viral RNA vector derived from a satellite virus" should at least include cis elements from a satellite virus which are recognized by an externally provided replicase, and an origin of assembly allowing encapsidation by the provided coat protein. Preferably, the viral RNA vector does not comprise a gene encoding a

functional coat protein, particularly it does not comprise the nucleotide sequence which is essentially similar to the nucleotide sequence encoding a coat protein gene. Particularly preferred are viral RNA vectors comprising an origin of assembly recognized by coat protein molecules from a rod-shaped virus, such as tobacco mosaic virus, since rod-shaped viruses do not exhibit the spatial constraints imposed on the size of genome by icosahedral viruses, thus allowing a larger number of additional nucleotides to be incorporated in the viral vector. Conveniently, the viral RNA vector comprises a number of unique or low-occurrence restriction recognition sites.

The use of viral RNA vectors derived from a satellite virus, additionally solves problems associated with the use of viral RNA vectors, such as reducing the size of the vectors, increasing versatility etc.

Particularly suited for the invention are viral RNA vectors derived from satellite tobacco mosaic virus comprising the origin of assembly (OAS) from TMV, preferably comprising the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 5443 to the nucleotide at position 5518 or the nucleotide of SEQ ID No 5 from the nucleotide at position 5430 to the nucleotide at position 5505 (such as the nucleotide sequence of SEQ ID No 12) and wherein the coat protein encoding gene has been deleted. Also particularly suited for the invention are viral RNA vectors derived from satellite necrosis vector strain comprising the OAS from TMV and wherein most of the coat protein gene has been deleted. Non-limiting examples of viral RNA vectors, suitable for the invention are described hereinafter.

"A corresponding helper virus" as used herein, indicates those RNA viruses, preferably single stranded RNA viruses, which can supply the satellite virus or the derived viral RNA vector with the functions required *in trans* by that satellite virus or the derived viral RNA vector, to allow it to replicate in the cytoplasm of plant cells, and spread throughout an infected plant. Typically, corresponding helper viruses will provide the satellite virus or the vector derived thereof with a replicase (RNA dependent RNA polymerase) which recognizes the *cis*



sequences present on the satellite virus RNA, and will allow replication of the satellite virus genome or the derived vector. Other proteins which may typically be provided by the helper virus are movement proteins, allowing inter alia, the plasmodesmata-mediated spread of viral particles from cell to cell. For satellite viruses or viral RNA vectors derived thereof which lack a functional coat protein encoding gene, corresponding helper viruses may also provide a functional coat protein. Preferably, the corresponding helper virus will be capable of autonomous systemic spread in an infected plant. However, such a systemic spread seems not to be a prerequisite for efficient gene silencing. Functions required *in trans* for one particular viral RNA vector may be supplied *in trans* by different corresponding helper viruses.

It is clear that the corresponding helper viruses may be wild type isolates of RNA viruses, preferably single-stranded RNA viruses such as the tobamoviruses or necroviruses. Particularly preferred are rod-shaped RNA viruses such as tobamoviruses including tobacco mosaic virus and the related tobamoviruses such as ribgrass mosaic virus, turnip vein clearing virus, chine rape mosaic virus, oilseed rape mosaic virus.

When TMV can be used as a helper virus, it can also be replaced by one of the closely related tobamoviruses mentioned above, particularly when using the viral vectors in particular plant species.

Also encompassed by the methods and means of the invention are variants of such wild type isolates, preferably variants or mutants which develop minimal symptoms when inoculated on host plants or when co-infected with a corresponding satellite virus or RNA vector derived thereof. Further preferred helper viruses may be variants or mutants of wild type isolates which have an extended host range such as tobamoviruses which can replicate and spread in corn or brassicae.

However, corresponding helper viruses may also be chimeric or hybrid viruses, wherein part of the viral genome has been replaced by a foreign nucleic acid, particularly wherein part of the viral genome has been replaced by a nucleic acid derived from another viral genome, preferably a part comprising a nucleotide sequence encoding a movement protein, or a part comprising a nucleotide sequence encoding a coat protein. E.g. when using a necrovirus such as TNV, it may be advantageous to insert a movement protein encoding region , preferably a movement protein derived from a tobamovirus such as TMV, particularly the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 4903 to the nucleotide at position 5709, so as to ensure spreading of the viral particles beyond the infected leaf. However, spreading of the helper virus or the viral RNA vector is not essential for efficient inactivation of expression of the target genes throughout the plant, as was found by the inventors. Also when using e.g. a necrovirus such as TNV, it may be further advantageous to replace the coat protein coding region of the necrovirus by a coat protein coding region of a rod-shaped virus, such as TMV, particularly the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 5712 to the nucleotide at position 6191. It goes without saying that an appropriate origin of assembly for the substituted coat protein has to be incorporated in the genome of the chimeric helper virus. In the above described example however, the OAS of TMV is conveniently located within the movement protein coding region. Non-limiting examples of corresponding helper viruses will be described hereinafter.

It should be clear that whenever it is stated that plants are co-infected or infected with a viral RNA vector and a corresponding helper virus, it is equal whether the helper virus is inoculated before, after or simultaneous with the viral RNA vector, provided however that there is a reasonable time limit between infection of the viral RNA vector or the corresponding helper virus.

Alternatively, the required functions *in trans* for the replication and movement of the viral RNA vector may be provided from the expression of chimeric genes, encoding a replicase (RNA dependent RNA polymerase) and/or a movement

protein and/or a functional coat protein, integrated in the genome of the test plants.

Preferred kits to deliver inhibitory RNA or gene-silencing constructs to plant cells to be used in the herein disclosed methods comprise a viral RNA vector derived from a satellite RNA virus, particularly from satellite tobacco necrosis vector (STNV) or satellite tobacco mosaic virus (STMV) and a corresponding helper virus, particularly a rod-shaped corresponding helper virus, wherein the viral RNA vector comprises a gene-silencing construct.

In a preferred embodiment the kit comprises a viral RNA vector derived from satellite tobacco necrosis vector, preferably comprising the cis-elements required for replication, particularly comprising the nucleotide sequence of SEQ ID No 3 from the nucleotide at position 1 to the nucleotide at position 32 and the nucleotide sequence of SEQ ID No 3 from the nucleotide at position 738 to the nucleotide at position 1245, wherein an origin of assembly of tobacco mosaic virus has been inserted, preferably comprising the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 5443 to the nucleotide at position 5518 or comprising the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 5430 to the nucleotide at position 5505, or comprising the nucleotide sequence of SEQ ID No 12 and wherein said helper virus is derived from tobacco necrosis virus, preferably with a nucleotide sequence of SEQ ID No 1, and comprises a gene encoding the movement protein of tobacco mosaic virus, preferably with the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 4903 to the nucleotide at position 5709 or with the nucleotide sequence of SEQ ID No 15 from the nucleotide at position 479 to the nucleotide at position 1285 and a gene encoding the coat protein of tobacco mosaic virus, preferably with the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 5712 to the nucleotide at position 6191 or with the nucleotide sequence of SEQ ID No 15 from the nucleotide at position 1288 to the nucleotide at position 1767.

Preferred combinations are those kits wherein the viral RNA vector is derived from STNV-1 or STNV-2 strains (as disclosed by Ysebaert et al. 1980; Genbank Accession number M10388 or Danthinne et al., 1991 Genbank Accession M64479) and the helper virus is TNV-A (Meulewaeter et al 1990, SEQ ID No 1).

- 5 Other preferred combinations are those kits wherein the viral RNA vector is derived from STNV-C (Bringloe et al. (1998); Genbank Accession Nr AJ000898) and the corresponding helper virus is TNV-D (Coutts et al. (1991); Genbank Accession Nr D00942).

- 10 In another particularly preferred embodiment the kit comprises a viral RNA vector derived from satellite tobacco mosaic virus, preferably comprising the cis-elements required for replication, particularly comprising the nucleotide sequence of SEQ ID No 4 from the nucleotide at position 1 to the nucleotide at position 197 and the nucleotide sequence of SEQ ID No 4 from the nucleotide at position 604 to the nucleotide at position 1058 or comprising the nucleotide sequence of SEQ ID No 13 and the nucleotide sequence of SEQ ID No 14; and  
15 further comprising an origin of assembly of tobacco mosaic virus, preferably comprising the nucleotide sequence of SEQ ID No 2 from the nucleotide at position 5443 to the nucleotide at position 5518 or comprising the nucleotide sequence of SEQ ID No 5 from the nucleotide at position 5430 to the nucleotide at position 5505 or comprising the nucleotide sequence from SEQ ID No 12, and  
20 wherein said corresponding helper virus is a tobacco mosaic virus, particularly TMV-U1 (SEQ ID No 2) or TMV-U2 (SEQ ID No 5).

- 25 It will be clear to the person skilled in the art that viral RNA vectors may be generated conveniently by *in vitro* transcription methods from cDNA copies of the viral RNA. Likewise, infectious viral RNA for the corresponding helper viruses may be generated from cDNA copies of their genome. Libraries, viral vectors and corresponding helper viruses may also be maintained by replication in plant  
30 cells.

Methods to infect or inoculate plants and plant cells with viral RNA vectors, helper viruses and libraries comprised within viral RNA vectors are well within in the realm of the person skilled in the art and may be performed according to the methods described in Walkey (1985).

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In one embodiment of the methods of the invention, plants are inoculated, e.g. with a solution containing the libraries of gene-silencing constructs in a viral vector, or with a solution containing a mixture of gene-silencing constructs in a viral vector and corresponding helper virus. The solution may further contain  
10 additional compounds to improve inoculation and infection of the plants, such as, but not limited to abrasives, adherents, tensio-active products and the like.

Plants may be infected during different developmental stages, in order to maximize the phenotype under investigation. Also different parts of plants may  
15 be inoculated to optimize observation of the expected phenotype.

Although not intending to limit the scope of the invention to a particular mode of action, it is thought that the inhibitory RNA comprised within the viral RNA vector can exercise its inhibiting effect, provided there is a balance between RNA  
20 encapsidated in a virion and free RNA. It is thought that the balance between encapsidated and free RNA may be influenced by varying the sequence and position of an origin of assembly within the viral RNA vector.

However, the gene-silencing effect may be amplified by placing the inhibitory RNA encoding nucleic acid under control of a viral promoter, preferably a coat  
25 protein promoter, or a subgenomic promoter so that during the life cycle of the virus additional inhibitory RNA is generated or transcribed.

"Gene-silencing constructs" as used herein is to be interpreted as a nucleic acid, which when transcribed yield "inhibitory RNA" comprising or consisting of sense  
30 RNA or antisense RNA, or a combination of both comprising a nucleotide sequence which has at least 75%, preferably at least 80%, particularly at least 85%, more particularly at least 90%, especially at least 95% sequence identity

with or is identical to the nucleotide sequence whose expression is to be suppressed, or its complement. Further, the nucleotide sequence of the sense or antisense region should preferably be at least about 100 nucleotides in length, more preferably at least about 250 nucleotides, particularly at least about 500  
5 nucleotides but may extend to the full length of the coding region of the gene whose expression is to be reduced.

For practical purposes in the application of the methods for high throughput screening or validation, the gene-silencing construct may be identical in  
10 sequence and length to the target nucleic acids, or they may be exactly complementary in sequence and identical in length to the target nucleic acids.

For the purpose of this invention the "sequence identity" of two related nucleotide or amino acid sequences, expressed as a percentage, refers to the  
15 number of positions in the two optimally aligned sequences which have identical residues (x100) divided by the number of positions compared. A gap, i.e. a position in an alignment where a residue is present in one sequence but not in the other is regarded as a position with non-identical residues. The alignment of the two sequences is performed by the Wilbur and Lipmann algorithm (Wilbur and Lipmann ,1983) using a window-size of 20 nucleotides or amino acids, a  
20 word length of 2 amino acids, and a gap penalty of 4. Computer-assisted analysis and interpretation of sequence data, including sequence alignment as described above, can be conveniently performed using commercially available software packages such as the programs of the Intelligenetics™ Suite  
25 (Intelligenetics Inc., CA) or the GCG Wisconsin Package.

It is clear for the person skilled in the art that the gene-silencing constructs may comprise at the same time sense and anti-sense RNA targeted towards the same nucleotide sequence whose expression is to be reduced. Preferably, the  
30 sense and antisense RNA are at least partly complementary to each other and capable of forming a stem-loop structure, since such a configuration has been shown to increase the efficiency of gene-silencing, both in occurrence and level

of gene-silencing (Waterhouse et al. 1998). In the most straightforward embodiment, at least part of the target nucleic acid, preferably the complete target nucleic acid, is cloned in duplicated form, whereby the two copies are in inverted repeat, preferably separated by an unrelated spacer nucleotide sequence.

The invention also aims at providing the herein described kits in their different embodiments. It is also an object of the invention to provide the kits comprising the helper viruses and viral RNA vectors described without the gene-silencing constructs or inhibitory RNA as well as their cDNA copies, whereby the cDNA copies are under control of a promoter (which can be used in *in vitro* transcription methods available in the art) such as but not limited to the promoters recognized by single subunit bacteriophage polymerase promoters (T7, T3, SP6 RNA polymerase specific promoters and the like).

The invention further relates to a method for identifying genes which are essential in plants comprising the following steps:

- a) A library of random gene-silencing constructs specific for the plant is created using a viral RNA vector which is derived from a satellite RNA virus, as herein defined including all its preferred embodiments. Preferably, the library is created in a cDNA copy of the viral vector and may be generated by inserting random DNA sequences, preferably at least about 100 nucleotides in length, particularly at least about 500 nucleotides in length. The random DNA sequences may be obtained from total DNA of a plant or may represent a subset of the genome of a plant, such as DNA derived from organelles (plastids, chloroplasts, mitochondria etc.) Alternatively, the library may be created by inserting cDNAs generated by reverse transcriptase from RNA, preferably mRNA obtained from said plant. The library may be normalized, e.g. as described in Takayuki et al. (1995). The library should preferably be large enough in size, i.e. contain a sufficient number of independent clones to cover the genome of the plant, according to the standards known in the art. In a preferred embodiment, the library may contain duplication of the

- inserted nucleic acid whereby the copies are in inverted repeat. The inserted nucleic acid may be cloned downstream of a viral promoter such as, but not limited to a coat protein gene promoter or a subgenomic promoter. It will further be clear to the person skilled in the art that the relative orientation of the inserted nucleic acid, in relation to the RNA vector is only of limited importance since either sense or antisense inhibitory RNA will be generated.
- 5 b) Assay plants are infected with individual members of the library and also with a corresponding helper virus. Infection may proceed according to any of the methods mentioned herein. Clearly, the DNA copy of the library should be converted into an RNA copy according to any of the methods described herein, preferably prior to the infection of the assay plants.
- 10 c) Plants developing a gene-silencing-construct-associated phenotype are identified. As used herein, a "gene-silencing-construct-associated phenotype" is meant to indicate a phenotype which is not observed when performing a mock inoculation with a viral RNA vector without gene-silencing construct, in combination with a corresponding helper virus on a similar plant. Preferred phenotypes comprise chlorosis, necrosis or any phenotype, preferably a morphological phenotype indicating that the infected tissue is inhibited or dying or deteriorating.
- 15 20d) Optionally, isolating the viral RNA vector from the tissue exhibiting the gene-silencing-construct-associated phenotype according to methods available in the art for isolation of virus. Preferably, the isolated viral RNA vector comprising the gene-silencing construct of interest should be re-assayed on fresh plants to confirm the observed phenotype. The viral RNA vector can of course also be recovered from the library if the infection of the plants was performed in an identity-preserving way.
- 25 e) The gene silencing construct or the nucleotide sequence information thereof, may then be used to recover the corresponding genomic or cDNA clone using methods available in the art (hybridization, PCR etc.)

As defined herein "essential genes" of a plant, are those genes which are necessary during the normal development of a plant. As defined, essential



genes may be essential for normal development only in particular developmental stages, or only in particular tissues or organs, such as e.g. flowers. Typically, inhibition of the expression of essential genes may have a lethal effect on a plant or part of a plant. Preferred essential genes are those genes which result in retardation or dying of seedlings when inhibited.

It will be clear for the person skilled in the art that if the inhibition of the target nucleic acid results in a dominant effect, as is the case for inhibition of the expression of essential genes, the described methods may be performed using infection of more than one viral RNA vector comprising a gene-silencing construct per plant. Care has to be taken to not dilute the phenotypic effect too much by infecting a too large number of different viral RNA vectors comprising differing gene-silencing constructs on the same plant. It is thought that optimally any number between one and five different inhibitory RNAs may be introduced in one plant cell.

In yet another embodiment of the invention, a method is provided for determining the function encoded by a nucleic acid comprising a known nucleotide sequence. This nucleotide sequence may have been obtained e.g. from a genome sequencing program, including expressed sequence tags sequencing programs. In order to unravel the function of that sequence, a gene-silencing construct or inhibitory RNA targeted towards said nucleotide sequence, as described in all its embodiments herein, may be introduced into a viral RNA vector derived from a satellite virus, as described herein, and used to inoculate a plant or being introduced into a plant cell, particularly into a protoplast, together with a corresponding helper virus, as described herein.

A large number of the embodiments described herein thus relate to a method for the introduction of inhibitory RNA in plant cells, comprising the steps of :

- 30a.) introducing into a plant cell, a viral RNA vector comprising inhibitory RNA or comprising a chimeric nucleic acid which when transcribed

yields the inhibitory RNA, wherein the viral RNA vector is derived from a satellite RNA virus; and

b.) introducing into the same plant cell, a corresponding helper virus.

5 The methods of the invention can be applied to essentially all plants for which viral vector and/or corresponding helper viruses are available. The methods of the invention are thought to be particularly suited for *Nicotiana* spp, particularly *N. tabacum*, *N. sylvestris*, *N. benthamiana*, and other *Solanaceae*, rice (*Oryza sativa*) corn (*Zea mays*), *Brassica* spp. , cotton (*Gossypium hirsutum*), wheat,  
10 *Arabidopsis* spp., *Petunia* spp.

Also envisioned by the present invention are methods for developing an agronomically useful product, such as a herbicide or a transgenic plant using the herein described methods and means, further comprising the steps of inserting  
15 a nucleic acid, involved in the determination of a particular plant trait, isolated by the methods of the invention, preferably under control of a foreign plant-expressible promoter, particularly under control of a controllable plant-expressible promoter into the genome of a plant , particularly a crop plant. When essential genes have been identified according to the methods described herein,  
20 these essential genes or their encoded gene products, particularly the encoded proteins may be used in *in vitro* assays to identify compounds inhibiting the activity, particularly the enzymatic activity, which may be used as herbicides. Alternatively, a viral RNA vector encoding gene-silencing constructs targeted towards essential genes may be used as herbicidal compounds.

25

The following non-limiting Examples describe the construction of viral RNA vectors derived from satellite viruses, and uses thereof. Unless stated otherwise in the Examples, all recombinant DNA techniques are carried out according to standard protocols as described in Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition, Cold Spring Harbor Laboratory Press, NY  
30 and in Volumes 1 and 2 of Ausubel *et al.* (1994) *Current Protocols in Molecular Biology*, Current Protocols, USA. Standard materials and methods for plant

molecular work are described in *Plant Molecular Biology Labfax* (1993) by R.D.D. Croy, jointly published by BIOS Scientific Publications Ltd (UK) and Blackwell Scientific Publications, UK.

- 5 Throughout the description and Examples, reference is made to the following sequences:

SEQ ID No 1: nucleotide sequence of the genome of TNV-A

SEQ ID No 2: nucleotide sequence of the genome of TMV-U1 ( Genbank  
10 Accession Nr V01408).

SEQ ID No 3: nucleotide sequence of the genome of STNV-2

SEQ ID No 4: nucleotide sequence of the genome of STMV (Genbank  
accession Nr. M25782).

SEQ ID No 5: nucleotide sequence of the genome of TMV-U2 ( Genbank  
15 Accession Nr M34077).

SEQ ID No 6: nucleotide sequence of the tomato phytoene desaturase (*pds*)  
encoding cDNA (Genbank Accession Nr. X59948).

SEQ ID No 7: nucleotide sequence of the tobacco nitrate reductase (*nia-2*)  
encoding cDNA (Genbank Accession Nr. X14059).

20 SEQ ID No 8: nucleotide sequence of the tobacco nitrite reductase (*nir-1*)  
encoding cDNA (Genbank Accession Nr. X66145).

SEQ ID No 9: nucleotide sequence of the  $\beta$ -1,3-glucanase( *gn-1*) encoding  
cDNA of *Nicotiana plumbagenifolia*.

SEQ ID No 10: nucleotide sequence of a green fluorescent protein (*gfp*)  
25 encoding region.

SEQ ID No 11: nucleotide sequence of a  $\beta$ -glucuronidase (*gus*) encoding region.

SEQ ID No 12: nucleotide sequence of an origin of assembly of a TMV-U2  
strain.

SEQ ID No 13: nucleotide sequence of the leader sequence of a STMV strain

30 SEQ ID No 14: nucleotide sequence of the trailer sequence of a STMV strain.

SEQ ID No 15: nucleotide sequence of part of the genome of a TMV-U2 strain  
comprising movement protein and coat protein genes.

## Examples

### Example I : construction of the viral RNA vector kits

5 A plasmid vector for the synthesis of an infective hybrid TMV/TNV helper virus RNA is made using the following operationally linked elements:

- A T7 RNA polymerase promoter
- A nucleic acid comprising the nucleotide sequence from the nucleotides 1 to 2234 of TNV-A (nt 1 to 2234 of SEQ ID No 1), wherein the AUG codon at  
10 nucleotides 2218-2220 mutated to a different codon
- A nucleic acid comprising the nucleotide sequence encoding the open reading frame for the movement protein of TMV-U1 (nt 4903-5709 of Genbank Accession Number V01408 or nt 4903-5709 of SEQ ID No 2 or nt 479-1285 of SEQ ID No 12)
- 15 • A nucleic acid comprising the nucleotide sequence from the nucleotides 2235 to 2612 of TNV-A (SEQ ID No 1)
  - A nucleic acid comprising the nucleotide sequence encoding the open reading frame for the coat protein of TMV-U1 (nt 5712-6191 of Genbank Accession Number V01408 or nt 5712-6191 of SEQ ID No 2 or nt 1288-  
20 1767 of SEQ ID No 15)
  - A sequence comprising nucleotide 3444 to 3684 of TNV-A (nt 3444 to 3684 of SEQ ID No 1)

A plasmid vector for the synthesis of an infective hybrid TMV/STNV viral vector  
25 RNA is made using the following operationally linked elements:

- A T7 RNA polymerase promoter
- A nucleic acid comprising the nucleotide sequence from nucleotide 1 to 32 of STNV-2 (nt 1 to 32 of SEQ ID No 3)
- A nucleic acid comprising the origin of assembly (OAS) of TMV-U1 (nt.  
30 5443-5518 of Genbank Accession Number V01408 or nt 5443-5518 of SEQ ID No 2 or nt 1018 to 1094 of SEQ ID No 15)

- A nucleic acid comprising the nucleotide sequence from nucleotide 738 to 1245 of STNV-2 (nt 738 to 1245 of SEQ ID No 3)

A plasmid vector for the synthesis of an infective hybrid TMV/STMV viral vector

5 RNA is made using the following operationally linked elements:

- A T7 RNA polymerase promoter
- A nucleic acid comprising the nucleotide sequence from nucleotide 1 to 197 of STMV (Genbank Accession Number M25782; nt 1 to 197 of SEQ ID No 4) or the nucleotide sequence of SEQ ID No 13.
- 10 • A nucleic acid comprising the OAS of TMV-U1 (nt. 5443-5518 of Genbank Accession Number V01408; nt 5443 to 5518 of SEQ ID No 2; nt 1019-1094 of SEQ ID No 15) or of TMV-U2 (nt 5430-5505 of Genbank Accession Number M34077; nt 5430-5505 of SEQ ID No 5) such as the nucleotide sequence of SEQ ID No 12.
- 15 • A nucleic acid comprising the nucleotide sequence from nucleotide 604 to 1058 of STMV (Genbank Accession Number M25782; nt 604 to 1058 of SEQ ID No 4) or comprising the nucleotide sequence of SEQ ID No 14.

## 20 **Example 2. Feasibility demonstration using known endogenes or transgenes.**

To demonstrate the feasibility of the use of the viral kits described sub example 1 for functional knockout of specific endo- or transgenes in *Nicotiana* plants, one of the following DNA fragments is inserted in the TMV/STNV or TMV/STMV hybrid vector, immediately upstream or downstream of the TMV OAS:

- a fragment of the tomato pythoene desaturase (*pds*) cDNA (comprising nucleotide 1021 to 1671 of SEQ ID No 6 or Genbank Accession Number X59948)
- a fragment of the tobacco nitrate reductase (*nia-2*) cDNA (comprising nucleotides 1103 to 2114 or nucleotides 5169 to 6497 of SEQ ID No 7 or
- 30 Genbank Accession Number X14059)

- a fragment of the tobacco nitrite reductase (*nir-1*) cDNA (comprising nucleotide 650-1212 of SEQ ID No 8 or Genbank Accession Number X66145)
- a fragment of the  $\beta$ -1,3-glucanase (*gn-1*) cDNA of *Nicotiana plumbaginifolia* (SEQ ID No 9 or Genbank Accession Number X07280)
- a fragment comprising a nucleotide sequence from a green fluorescent protein (*gfp*) coding region (SEQ ID No 10)
- a fragment comprising a nucleotide sequence from nucleotide 1 to 600 of the  $\beta$ -glucuronidase (*gus*) coding region (SEQ ID No 11)

10

Infective chimeric transcripts are synthesized *in vitro*, using T7 RNA polymerase with the linearized plasmid DNAs of the described vectors as templates.

15

The TMV/STNV RNAs are mechanically inoculated on leaves of *Nicotiana benthamiana* or *Nicotiana tabacum* plants together with the TMV/TNV RNA, whereas the TMV/STMV RNAs are inoculated together with TMV-U2 virus particles or viral RNA.

The infected plants are scored for phenotypes, virus accumulation, and suppression of the homologous plant gene between 1 and 4 weeks after inoculation.

20

Plants infected with vectors containing the *pds* cDNA show a bleaching phenotype on infected leaves and silencing of the endogenous *pds* transcript. Plants infected with vectors containing the *nia-2* or *nir-1* cDNA show a chlorotic phenotype on infected leaves and silencing of the endogenous *nia-2* or *nir-1* transcript, respectively.

25

Plants infected with vectors containing the *gn-1* cDNA show silencing of the endogenous basic -1,3-glucanase transcript.

Upon infection with vectors containing the *gfp* sequence, transgenic plants that normally express a *gfp* transgene show silencing of the *gfp* transgene transcript and suppression of GFP fluorescence.

30

Upon infection with vectors containing the *gus* sequence, transgenic plants that normally express a *gus* transgene show silencing of the *gus* transgene transcript and suppression of GUS activity.

**Example 3: Inactivation of phytoene desaturase in *Nicotiana benthamiana* using a TNV/STNV hybrid vector system.**

A TNV/STNV hybrid vector system was used for the functional inactivation of a constitutively expressed endogenous plant gene . Therefore, the following STNV hybrid vectors have been constructed:

10

pIF9 carrying the following operationally linked elements:

- a T7 RNA polymerase promoter comprising nucleotide 402 to 420 of Genbank Accession Number M77811;
- a nucleic acid comprising the nucleotide sequence from nucleotide 1 to 32 of SEQ ID No 3 (STNV-2 leader);
- a nucleic acid comprising the origin of assembly (OAS) of TMV-U1 from nucleotide 5443 to 5518 of SEQ ID No 2 or Genbank Accession Number V01408;
- a fragment of the tomato phytoene desaturase (pds) cDNA comprising nucleotide 1021 to 1671 of SEQ ID No 6 or Genbank Accession Number X59948;
- a nucleic acid comprising the nucleotide sequence from nucleotide 806 to 1418 of SEQ ID No 3 (STNV-2 trailer).

25

pIF12 carrying the following operationally linked elements:

- a T7 RNA polymerase promoter comprising nucleotide 402 to 420 of Genbank Accession Number M77811;
- a nucleic acid comprising the nucleotide sequence from nucleotide 1 to 32 of SEQ ID No 3 (STNV-2 leader);
- a fragment of the tomato phytoene desaturase (pds) cDNA comprising nucleotide 1021 to 1671 of SEQ ID No 6 or Genbank Accession Number X59948;

30

- a nucleic acid comprising the nucleotide sequence from nucleotide 742 to 1354 of SEQ ID No 3 (STNV-2 trailer).

Infective chimeric transcripts have been synthesized in vitro using T7 RNA polymerase with the linearized plasmid DNAs of the described pIF9 and pIF12 hybrid vectors as templates using standard procedures. Control in vitro transcripts have been synthesized on linearized plasmid DNAs of the precursor plasmids of pIF9 and pIF12 without the pds fragment inserts and on linearized plasmid DNA of an infective clone of the STNV wild type and of a hybrid STNV vector carrying an insert of the cat gene.

The in-vitro transcripts have been mechanically inoculated onto leaves of four weeks old *Nicotiana benthamiana* plants together with the TNV helper virus.

All infected plants were continuously scored for pds inactivation, which resulted in a phenotype showing leaf bleaching. For all inoculations necrotic lesions were observed for the inoculated leaves after 2 days post inoculation (p.i.). Within a week, necrotic lesions also occurred in upper leaves indicating the systemic spread of the viruses in *N.benthamiana*. In most infected plants, the virus symptoms have been severe but plants survived for many weeks.

Only plants, which have been infected with the hybrid vectors pIF9 and pIF12 carrying pds fragments, showed on top of the virus symptoms additional phenotypic changes. Approximately 4 weeks p.i., green upper leaves, which did not show any virus symptoms, developed bleached spots scattered all over the leaves. The bleaching was progressive and was not accompanied by necrotic lesions. Within another three weeks, the size of the spots increased constantly and the color changed from pale green to yellow to pale white-yellow. These symptoms have never been observed in plants, on which TNV/STNV wild type or TNV/STNV-deletion mutant control inoculations were carried out. Thus, this phenotype indicates the functional knockout of pds in *N. benthamiana* plants after infection with pIF9 and pIF12 hybrid viral vectors.



In RNA gel blot analyses performed with total RNA preparations of green and bleached spots of upper leaves of plants showing a FKO phenotype no chimeric STNV virus RNA and only very low levels of TNV helper virus RNA could be detected. This indicates that virus induced gene silencing of pds in a specific tissue does not need to be accompanied by high levels of virus RNA.

**Example 4: Inactivation of phytoene desaturase in *Petunia hybrida* using a TMV/STMV hybrid vector system.**

A TMV/STMV hybrid vector system was used for the functional inactivation of a constitutively expressed endogenous plant gene. Therefore, the following STMV hybrid vectors have been constructed:

pVE293 carrying the following operationally linked elements:

- 15• a T7 RNA polymerase promoter comprising nucleotide 402 to 420 of Genbank Accession Number M77811
  - a nucleic acid comprising the nucleotide sequence from nucleotide 1 to 197 of STMV of SEQ ID No 4 or Genbank Accession Number M25782 (STMV leader)
- 20• a nucleic acid comprising the origin of assembly (OAS) of TMV-U2 from nucleotide 5430 to 5505 of SEQ ID No 5 or Genbank Accession Number M34077 ("short" TMV OAS)
  - a fragment of the tomato phytoene desaturase (pds) cDNA comprising nucleotide 1021 to 1671 of SEQ ID No 6 or Genbank Accession Number
  - 25 X59948
    - a nucleic acid comprising the nucleotide sequence from nucleotide 604 to 1058 of STMV of SEQ ID No 4 or Genbank Accession Number M25782 (STMV trailer)
    - a SP6 RNA polymerase promoter comprising nucleotide 143 to 124 of
    - 30 Genbank Accession Number X65308

pVE294 carrying the following operationally linked elements:

- a T7 RNA polymerase promoter comprising nucleotide 402 to 420 of Genbank Accession Number M77811
- a nucleic acid comprising the nucleotide sequence from nucleotide 1 to 197 of STMV of SEQ ID No 4 or Genbank Accession Number M25782 (STMV leader)
- a nucleic acid comprising the origin of assembly (OAS) of TMV-U2 from nucleotide 5441 to 5849 of SEQ ID No 2 or Genbank Accession Number M34077 ("long" TMV OAS)
- 10• a fragment of the tomato phytoene desaturase (pds) cDNA comprising nucleotide 1021 to 1671 of SEQ ID No 6 or Genbank Accession Number X59948
- a nucleic acid comprising the nucleotide sequence from nucleotide 604 to 1058 of STMV of SEQ ID No 4 or Genbank Accession Number M25782
- 15 (STMV trailer)
- a SP6 RNA polymerase promoter comprising nucleotide 143 to 124 of Genbank Accession Number X65308

20 Infective chimeric transcripts have been synthesized in vitro using T7 RNA polymerase with the linearized plasmid DNAs of the described pVE293 and pVE294 hybrid vectors as templates using standard procedures. Control in vitro transcripts have been synthesized on linearized plasmid DNAs of the precursor plasmids of pVE293 and pVE294 without the pds fragment insert and on linearized plasmid DNA of the infective clone STMV-10 of the wild type.

25

The in vitro transcripts have been mechanically inoculated onto leaves of three months old *Petunia hybrida* V26 plants together with the TMV-U2 helper virus.

30 The infected plants have been continuously scored for pds inactivation, which resulted in a phenotype showing leaf bleaching. The infection of petunia plants with TMV/STMV caused the occurrence of crinkled leaves starting from the infected branches but quickly progressing systemically throughout the plant. The

plants did survive the infections and often showed recovery phenotypes with almost no symptoms.

Only the plants, which have been infected with the hybrid vectors pVE293 and pVE294 carrying the pds fragment, showed in addition to the virus symptoms, additional phenotypic changes. In case of TMV/pVE293 infections, the infected branches developed young leaves with bleached sectors around the veins and at the tip of the leaves. This bleaching was progressive and independent from the presence of virus symptoms. Some of the leaves were fully bleached and progressively changed into almost white color. This phenotype was restricted to leaves of the infected branches. In case of TMV/pVE294 infections a similar bleaching of young leaves was observed for all developing branches but not for branches carrying already terminal flower buds. The bleaching started again around the veins and leaf tips but proceeded quickly producing variable leaf variegation patterns. This phenotype has never been observed in control inoculations with wild type viruses or deletion mutants. Therefore this phenotype indicates the functional knockout of pds in *Petunia hybrida* plants after infection with pVE293 and pVE294 viral hybrid vectors.

In RNA gel blot analyses performed with total RNA preparations of green and bleached spots of upper leaves of plants showing a FKO phenotype no chimeric STMV virus RNA and only low levels of TMV helper virus RNA could be detected. This indicates that virus induced gene silencing of pds in a specific tissue does not need to be accompanied by high levels of virus RNA.

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